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Identification of a Nucleocapsid Protein (VP35) Gene of Shrimp White Spot Syndrome Virus and Characterization of the Motif Important for Targeting VP35 to the Nuclei of Transfected Insect Cells

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To identify the protein encoded by a 687-bp open reading frame (ORF) of a *sal*I genomic DNA fragment of shrimp white spot syndrome virus (WSSV), we expressed the ORF in a baculovirus/insect cell expression system. The apparent molecular mass of the recombinant protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was 35 kDa in insect cells. Antibody raised against bacterially synthesized protein of the ORF identified a nucleocapsid protein (VP35) in the extracts of both the purified WSSV virions and the nucleocapsids which comigrated with the 35-kDa baculovirus-expressed recombinant protein on SDS-PAGE. We also show by transient expression in insect cells (Sf9) that VP35 targets the nucleus. Two potential nuclear localization signals (NLSs) were characterized, but only one of them was important for targeting VP35 to the nuclei of transfected insect cells. Replacement of a cluster of four positively charged residues (^{24}KRKR^{27}) at the N terminus of the protein with AAAA resulted in mutant proteins that were distributed only in the cytoplasm, thus confirming that this sequence is a critical part of the functionally active NLS of VP35.

Key Words: Penaeus monodon; white spot syndrome virus (WSSV); nucleocapsid; nuclear localization signal (NLS); VP35.

INTRODUCTION

White spot syndrome virus (WSSV) is the causative agent of a disease that has led to severe mortalities of cultured shrimps in Taiwan and many other countries (Chou *et al.,* 1995; Flegel, 1997; Lo *et al.,* 1999). WSSV is an enveloped, ellipsoid, large, double-stranded DNA virus (Wang *et al.,* 1995; Wongteerasupaya *et al.,* 1995) and it has a wide host range among crustaceans (Lo *et al.,* 1996, 1998). Even while the molecular data was still limited, the morphological characteristics and general biological properties of the virus (Wang *et al.,* 1995; Wongteerasupaya *et al.,* 1995; Lo *et al.,* 1996) already highlighted its uniqueness, and recent data, including the genome sequence and phylogenies based on DNA polymerase and protein kinase, suggest that WSSV is a member of a new virus family (van Hulten *et al.,* 2001; Liu *et al.,* 2001). The size of the WSSV genome has been differently reported for different isolates: 305107 bp (AX151396), 292967 bp (van Hulten *et al.,* 2001) and 307287 bp (AF440570) for viruses isolated from China, Thailand, and Taiwan, respectively, with these size differences mostly being due to several small insertions and one large (~12 kb) deletion. The uniqueness of this virus means that a thorough study of its molecular biology is still urgently needed for a better understanding of its nature and replication strategy and the molecular mechanisms of its pathogenesis.

All animal DNA viruses, except poxviruses (Wittek, 1982) and irridovirus (Tidona and Darai, 1997), replicate in the cell nucleus (Kasamatsu and Nakanishi, 1998). As a first step in infection, viruses import their genomic DNA into the nuclei of infected cells, and it is here that transcripts leading to viral protein synthesis are made. Most viruses utilize the nuclear import system of the cell, including the microtubules (Sodeik *et al.,* 1997; Suomalainen *et al.,* 1999), the nuclear pore complex (Greber *et al.,* 1996, 1997), receptors, and import factors (Marsh and Helenius, 1989; Whittaker and Helenius, 1998; reviewed in Kasamatsu and Nakanishi, 1998) to access the nucleus. Often, viruses that are too large to easily enter the nucleus will first locate to the nuclear pore and then release their DNA for transport to the nucleus in association with one or more mediating viral proteins. Thus for example, canine parvovirus (Vihinen-Ranta *et al.,* 2000), hepatitis B virus (Kann *et al.,* 1999), adenovirus (Greber *et al.,* 1997), and simian virus 40 (Wychowski *et al.,* 1986, 1987; Nakanishi *et al.,* 1996) all use one or several viral proteins to mediate the import of their viral DNA into the nucleus. In a normal eukaryotic cell, for a cellular protein to pass through the central transporter channel into the nucleus, it must contain some kind of

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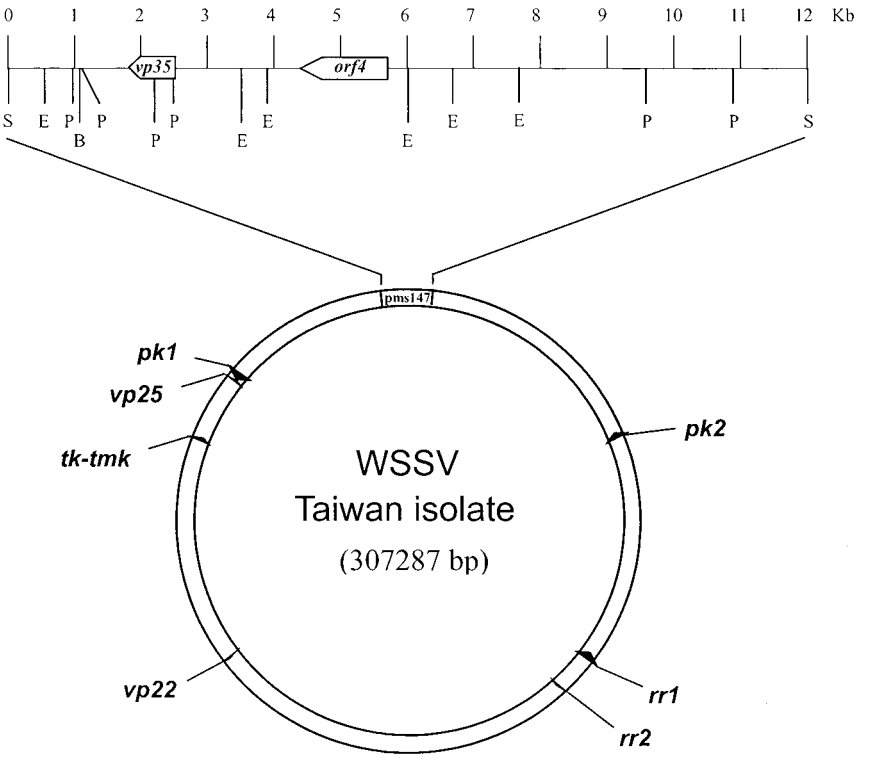

FIG. 1. Restriction enzyme map of the pms147 *Sal*I (S) fragment of WSSV genomic DNA showing the location (nt 1818 to nt 2504) of the WSSV *vp35* gene (pms147 ORF5). Because a pms147 *orf4*-specific riboprobe is used later for Northern blotting analysis, the *orf4* gene is also shown for reference. The pms147 segment contains six *Pst*I (P) sites, six *Eco*RI (E) sites, and one *Bam*HI (B) site. The relative positions on the entire WSSV genome for this fragment and for other published genes [*rr* genes (Tsai *et al.,* 2000a); *tk-tmk* (Tsai *et al.,* 2000b); *vp22, vp25,* and *pk1* (Liu *et al.,* 2001)] are also shown for reference. A protein kinase gene (*pk2*) that is distinctly related to the published protein protein kinase (*pk1*) is also indicated.

nuclear localization signal (NLS); likewise if a viral protein targets the nucleus, whether it acts to mediate the import of viral DNA or not, it too must include an NLS (Kalderon *et al.,* 1984; reviewed in Jans and Hubner, 1996, and in Kasamatsu and Nakanishi, 1998). The classical versions of this signal consist of predominantly basic amino acids appearing in either one (simple type or monopartite NLS) or two (bipartite NLS) short clusters.

WSSV structural proteins are unique in that they do not show any obviously significant bands in SDS-PAGE, and from the WSSV genome sequence, no genes have yet been identified that show high homology to the structural protein genes of baculoviruses or other DNA viruses. It is therefore hard to directly apply other virus infection models to interpret the infection strategy of WSSV; on the contrary, WSSV's infection strategy must be investigated *ab initio.* Thus, because WSSV structural protein(s) that have an NLS might play a role in mediating the import of WSSV DNA into the nuclei of infected cells, we began by focusing on structural proteins that exhibit nuclear targeting behavior. WSSV VP35 is one such structural nucleocapsid protein. In the present paper, we identify and characterize its ORF in a WSSV *sal*I genomic DNA fragment and express the protein in a baculovirus/insect system. We also use transient expression in insect cells (Sf9) to show that VP35 targets the nucleus, and define and characterize a cluster of four positively charged residues at the N-terminus of VP35 that directs the protein to the nucleus. This report is the first description of a functionally active NLS within a nucleocapsid protein of shrimp white spot syndrome virus.

RESULTS AND DISCUSSION

Location and sequence of the ORF encoding VP35

The virus used in this study was isolated from a batch of WSSV-infected *Penaeus monodon* collected in Taiwan in 1994 (Wang *et al.,* 1995), which is now known as WSSV Taiwan isolate (Lo *et al.,* 1999). From this virus, a plasmid library (referred to as the pms library, where pm indicates *Penaeus monodon* and s indicates *Sal*I fragments) of WSSV *Sal*I genomic fragments was constructed (Wang *et al.,* 1995). The pms147 ORF5 (located at nt 1818 to 2504 of the 12002 bp pms147) was used in the present study (Fig. 1). This ORF encodes a protein of 228 amino acids with a theoretical size of 26 kDa and an isoelectric point of 4.51.

The nucleotide sequence surrounding the methionine start codon (AAA**ATG**G; see Fig. 2) of the predicted protein conformed to the Kozak rule of an efficient context for eukaryotic translation initiation (Kozak, 1989). A polyadenylation signal (AATAAA) was located 7 nt down-

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GTGTATATATAGGACCCCCGATCCTGCACCACAGCACACTTGATCATCGTAATCATCAGCATCCTAACCTAATACTACTACTACTTGTTCTACCCACGAGTTCCAAAAATAACCCCCAAA   -1
ATGGTCTCTTCTAGAACATCAACAACATCTTCATCTGCAGTAGCAGCCACCTCTACTCTTCTCCCCACCAAGAGGAAGAGGGAGCCAGAAGAAGTAAAGGTGAAAGTGGAAGTAAAAATG   120
M  V  S  S  R  T  S  T  T  S  S  S  A  V  A  A  T  S  T  L  L  P  T  K  R  K  R  E  P  E  E  V  K  V  K  V  E  V  K  M    40

GAACAAGAAGAACTGGTAGAGGACTCGTCAAGTAACAAGCGCCCCAGAATTAACGGAAGAGAAGGAGGAAGAACACAAAGAAACACATCACCTCTCCCTCCCATGTAAAGAAGAAGAAGAC   240
E  Q  E  E  L  V  E  D  S  S  S  N  K  R  P  R  I  K  E  E  K  E  E  E  H  K  E  T  H  H  L  S  L  P  C  K  E  E  E  D    80

GATGGTGAAGAAGAGGAATATGAGGAAGAGGAGGATGAGGAAGAATATGAAGACAGAGTGGACGACGACACTGCAGAGAAAATGGAAAATCTTTTGGTCCAACTGGACAATACTACCAAA   360
D  G  E  E  E  E  Y  E  E  E  E  D  E  E  E  Y  E  D  R  V  D  D  D  T  A  E  K  M  E  N  L  L  V  Q  L  D  N  T  T  K    120

AACATCAAACTGAAAAACCCCCTAAGGGAACATGACATGGCAGTTTCACACTATGAGCATGAATTTGAGGTACAAAATACTGTCAATTTTAGTTTTGGAGTACTATCTGATATTGGGTTC   480
N  I  K  L  K  N  P  L  R  E  H  D  M  A  V  S  H  Y  E  H  E  F  E  V  Q  N  T  V  N  F  S  F  G  V  L  S  D  I  G  F    160

CTGATCAACCGTGAAGCCGTTTCTAGGTGGGGTAATACACCCCCACCAAAAGAGTTTGGCGACATGGAGATTGGATCTCTTACAGTTAACCAGTTGCTCCACAAGTGTGATAATTTTGTA   600
L  I  N  R  E  A  V  S  R  W  G  N  T  P  P  P  K  E  F  G  D  M  E  I  G  S  L  T  V  N  Q  L  L  H  K  C  D  N  F  V    200

CAGGCTGTAGTACAGAAAGTGAAGGAAGATATAACCCCTTCTATTGAAGTTACAATAGATAGTTTGATTGATGATCCTTGTTGGTAAAAAACAAATAAAATATACCTAAAAATTATTCCA   720
Q  A  V  V  Q  K  V  K  E  D  I  T  P  S  I  E  V  T  I  D  S  L  I  D  D  P  C  W  *        *****                    228
                                                                                                poly adenylation signal
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FIG. 2. The nucleotide sequence and deduced amino acid sequence of the WSSV *vp35* gene. The two potential NLSs (boxes) and the acidic amino acid-rich domain (shaded) are indicated. The polyadenylation signal is marked with asterisks.

stream of the translational stop codon. Based on the PROSITE database, the potential sites for posttranslational modifications of this protein include two sites (NTTK[120] and NFSF[152]) for N-linked glycosylation, four sites (SSR[5], TKR[25], SNK[53], and TTK[120]) for PKC phosphorylation, two sites (SHYE[139] and SLID[224]) for CKII phosphorylation, and two sites (KRKR[27] and KRPR[56]) for potential endoprotease cleavage. Computer analysis of the protein showed that at the N terminus, there is quite a large continuous hydrophilic region that includes the acidic amino acid-rich domain shown in Fig. 2. The pms147 ORF5 protein was subsequently identified as a nucleocapsid protein by the following experiments.

Expression of the pms147 ORF5 (VP35) DNA in eukaryotic cell-free system

An expression vector, pcDNA3-HA-VP35, with the full length (nt 1818 to nt 2504) of pms147 ORF5 DNA was constructed for coupled *in vitro* transcription and translation reactions in a cell-free system with rabbit reticulocyte lysate. The translated protein was expected to be a hemagglutinin epitope (MCYPYDVPDYASLA)-tagged polypeptide (HA-tagged VP35). Following the TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI) protocols, a single protein band visualized by autoradiography was synthesized. This had an apparent molecular mass higher than the theoretical size of the ORF5 protein and was about 35 kDa on SDS-PAGE (Fig. 3A). This polypeptide was specifically detectable with a commercial antibody against the tag (data not shown) and also was recognized by antibody raised against bacterially synthesized ORF protein (Fig. 3B). Thus, we conclude that the pms147 ORF5 protein expressed in the eukaryotic cell-free translation system had an apparent molecular mass of 35 kDa on SDS-PAGE. However, since the apparent size of this pms147 ORF5 *in vitro* translation product was significantly higher than the calculated molecular mass of 26,000, further verifications were made.

Expression of the pms147 ORF5 DNA in a baculovirus/insect cell systems

Using the BacVector Transfection system, an *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV)-based recombinant virus was constructed for the expression of the pms147 ORF5 in Sf9 cells. To this end, a recombinant AcMNPV (VL1392-VP35) with the full length of the pms147 ORF5 was constructed and propagated in Sf9 cells at 27°C in Sf-900 II SFM cell culture medium (Gibco BRL). The recombinant protein (rVP35;

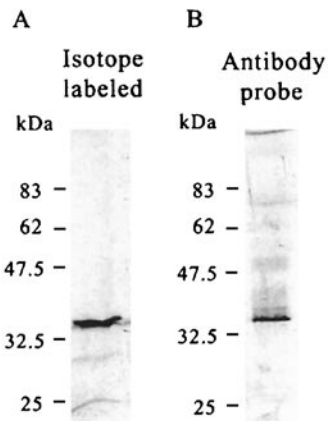

FIG. 3. VP35 yielded in eukaryotic *in vitro* translation system visualized by (A) autoradiography and (B) Western blot analysis. The size standards were determined using BenchMark prestained protein ladder (Gibco BRL) and were corrected with BenchMark protein ladder (Gibco BRL).

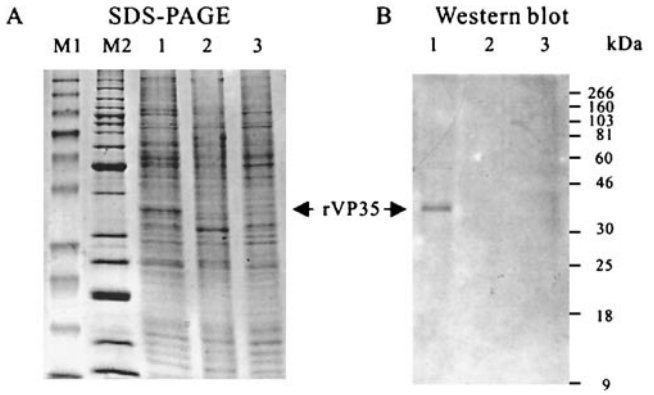

FIG. 4. Detection of the recombinant VP35 (rVP35) in insect cells by Coomassie blue staining (A) and Western blotting (B). Lane 1: Cell lysate proteins from the Sf9 cells infected with recombinant AcMNPV (VL1392-VP35). Lane 2: Cell lysate proteins from the Sf9 cells infected with wild-type AcMNPV. Lane 3: Cell lysate proteins from the uninfected Sf9 cells. M1: BenchMark prestained protein ladder. M2: BenchMark protein ladder. The size standards indicated in (B) were based on prestained protein ladder corrected with BenchMark protein ladder.

fusion protein of VP35 with additional 13 amino acids at N terminus) synthesized in insect cells infected with recombinant baculovirus VL1392-VP35 had an apparent molecular mass of 35 kDa on SDS-PAGE (Fig. 4A) (i.e., it was equal in size to the protein detected in the eukaryotic cell-free *in vitro* translation system) and it was recognized by antibody raised against the bacterially synthesized pms147 ORF5 protein (Fig. 4B).

It is well documented that very acidic proteins such as the constitutive nucleolar protein (NO29; pI 3.75) of *Xenopus laevis* protein (Zirwes *et al.,* 1997) and brain acid-

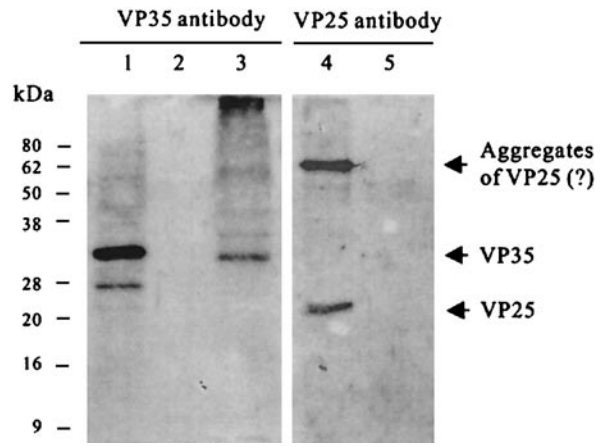

FIG. 5. Analysis of VP35 localization by SDS-PAGE and Western blotting. Lane 1: Cell lysate proteins from the Sf9 cells infected with recombinant AcMNPV (VL1392-VP35). Lanes 2, 4: Envelop fraction of the purified WSSV virion. Lanes 3, 5: Nucleocapsid fraction of the purified WSSV virion. Proteins were separated on 12.5% SDS-PAGE and then probed with VP35 antiserum (lanes 1, 2, and 3) or VP25 (envelope protein) antiserum (lanes 4 and 5). The size standards were determined using BenchMark prestained protein ladder and were corrected with BenchMark protein ladder.

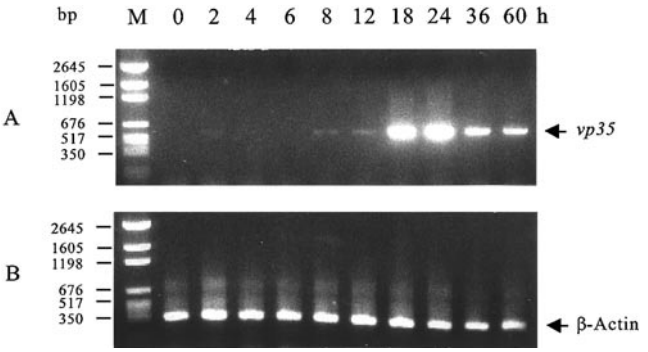

FIG. 6. Temporal transcription analysis of WSSV by RT-PCR. (A) RT-PCR with *vp35*-specific primers. (B) Internal positive control: total RNA treated with DNase and amplified with actin-specific primers. The products were resolved in 1% agarose gel containing 0.5 μg/ml ethidium bromide. The size standards are indicated using pGEM DNA marker (Promega). Lane headings show hours pi.

soluble proteins (BASPs; pI 4.4–4.6) (Mosevisky *et al.,* 1994) show an abnormally low mobility during SDS-PAGE electrophoresis. That is, they all show a SDS-PAGE mobility that corresponds to a molecular mass higher than the calculated molecular mass. This phenomenon presumably accounts for the difference between the calculated molecular mass and apparent molecular mass on SDS-PAGE of VP35 (pI 4.51).

Identification of the pms147 ORF5 protein as a nucleocapsid protein

Antibody raised against bacterially synthesized protein of the pms147 ORF5 identified a nucleocapsid protein (VP35) in the extracts of the purified WSSV virions which comigrated with the 35-kDa baculovirus-expressed recombinant protein on SDS-PAGE (Fig. 5, lane 1). VP35 was further shown to associate with the nucleocapsid fraction, but not with envelope fraction, in a triton-treated preparation of the purified virions (Fig. 5, lanes 2 and 3). Conversely, antibody against VP25 (a WSSV envelope protein) reacted with protein in the envelope fraction, but did not react with the nucleocapsid fraction (Fig. 5, lanes 4 and 5). These results confirm that VP35 is a WSSV nucleocapsid protein.

Transcriptional analysis of *vp35*

To discover when the *vp35* mRNA is transcribed in the viral life cycle, and which mRNA is transcribed, we performed a transcriptional analysis of *vp35* using RT-PCR and Northern blot analysis. Due to the lack of a shrimp cell line, it is impossible to do a temporal analysis of WSSV gene transcription in cells synchronously infected with the virus. However, WSSV is extremely virulent, and the onset of the disease is remarkably rapid in infected shrimp (Chou *et al.,* 1995). Further, the disease progresses at a more or less similar pace in different individuals, especially in cuticular epithelial and connec-

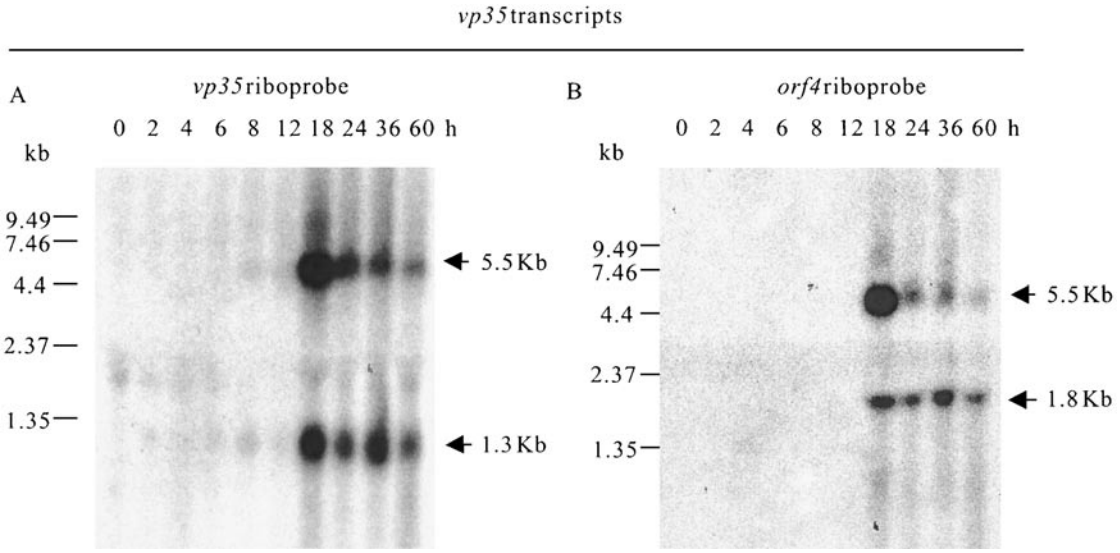

FIG. 7. Northern blot temporal transcription analysis of total RNA isolated from WSSV-infected *P. monodon* using *vp35*- and pms 147 *orf4*-specific riboprobes. (A) Two transcripts of *vp35* are approximately 5.5 and 1.3 kb. (B) Two transcripts of pms 147 *orf4* are approximately 5.5 and 1.8 kb. The size standards were determined by RNA marker (Gibco BRL). Lane headings show hours pi.

tive tissues. We therefore performed the transcriptional analysis in experimentally infected shrimp using tissues from the pleopod as the source of total RNA, because this organ most frequently showed the highest prevalence of the virus in early infection (Lo *et al.,* 1997; Kou *et al.,* 1998).

RT-PCR analysis revealed that the *vp35* transcript was first detected at 2 h p.i. and continued to be expressed at a very low level through to 12 h p.i. (Fig. 6A). The *vp35* RT-PCR product then significantly increased at 18 and 24 h p.i. and declined again at 36 h p.i. (Fig. 6A). Northern blot analysis with *vp35* specific riboprobe first detected two major transcripts of approximately 5.5 and 1.3 kb at 18 h p.i. (Fig. 7A). The temporal expression pattern of the 1.3 kb transcript (Fig. 7) is very similar to the pattern observed in the RT-PCR analysis (Fig. 6), and the length of the 1.3-kb transcript matched well with the length of the *vp35* coding region (687 bp) (assuming 5′ and 3′ UTRs totally of several hundred nucleotides). The 5.5 kb band (Fig. 7) was very intense at 18 h p.i. and then dropped significantly. A riboprobe for *orf4* located 3 kb upstream of *vp35* in the pms 147 fragment (see Fig. 1) also hybridized with the 5.5 kb transcript (Fig. 7B), indicating that this transcript must have been transcribed from a region in the pms 147 fragment that starts upstream of the *orf4* coding region and extends downstream to *vp35.* The difference in intensities of the 1.3-kb transcript and the 5.5-kb transcript at 18 h p.i. also suggests that WSSV uses different promoters (with different strengths) to initiate transcription of the two *vp35*-containing (5.5 and 1.3 kb) transcripts. It is also worth noting that because the primer set used in RT-PCR was located in the *vp35* coding region, Fig. 6A shows a combined result amplified from both the 1.3 kb transcript and the 5.5 kb transcript.

Identification of VP35 protein nuclear localization signal (NLS)

Inspection of the 228 amino acids of VP35 revealed two clusters of four amino acid residues (^{24}KRKR^{27}, ^{53}KRPR^{56}) at the N terminus of the protein. These motifs resemble many of the characterized classical NLSs, which are made essentially of a short stretch of positively charged amino acids (Fig. 8). We therefore investigated whether these two sequences function as the nuclear localization signal(s) of VP35.

```
Protein/virus                          :     NLS motif
VP35(NLS1)/WSSV                        : -----KRR-R--------
VP35(NLS2)/WSSV                        : -----KRPR---------
ICP4/Herpes simplex virus 1            : -----GRRKSP-------
ICP0/Herpes simplex virus 1            : ---VRPRRRGS-------
UL3/Herpes simplex virus 2             : ------RPRK--------
MEQ/Merk's disease virus               : -----KRKRK--------
ICP22/Equine herpesvirus               : ----KRKR----------
E1a/Adenovirus                         : -DLSCRPR-P--------
pTP/Adenovirus                         : -RLPVRRRVP-------
L1/Human papilloma virus16             : -----KRKRK--------
L2(5-11)/Human papilloma virus16       : ---RARKR----------
L2(444-449)/Human papilloma virus16    : ----RRKR----------
Large T/Simian virus 40                : ---P-KRKV--------
Vp2/3/Simian virus 40                  : ---PNKRKL--------
Vp1/Murine polyomvirus                 : --AP-RR-----------
Vp2/3/Murine polyomvirus               : ---PQKRKL--------
Vp2/Avian polyomvirus                  : --VP-RRKLPT------
Large T/human papovavirus BK virus     : ---P-KRKV--------
Core protein(145-156)/Hepatitis B virus: ETTVVRRGRSP-------
Core protein(172-183)/Hepatitis B virus: ----RRSQSRESQC----
EBNA-1/Epstein-Barr virus              : ----LRPRSPSS------
EBNA-3a/Epstein-Barr virus             : --RDRRNPASR-------
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FIG. 8. Alignment of the deduced NLS amino acid sequences of WSSV VP35 with the NLS motifs of other DNA virus proteins.

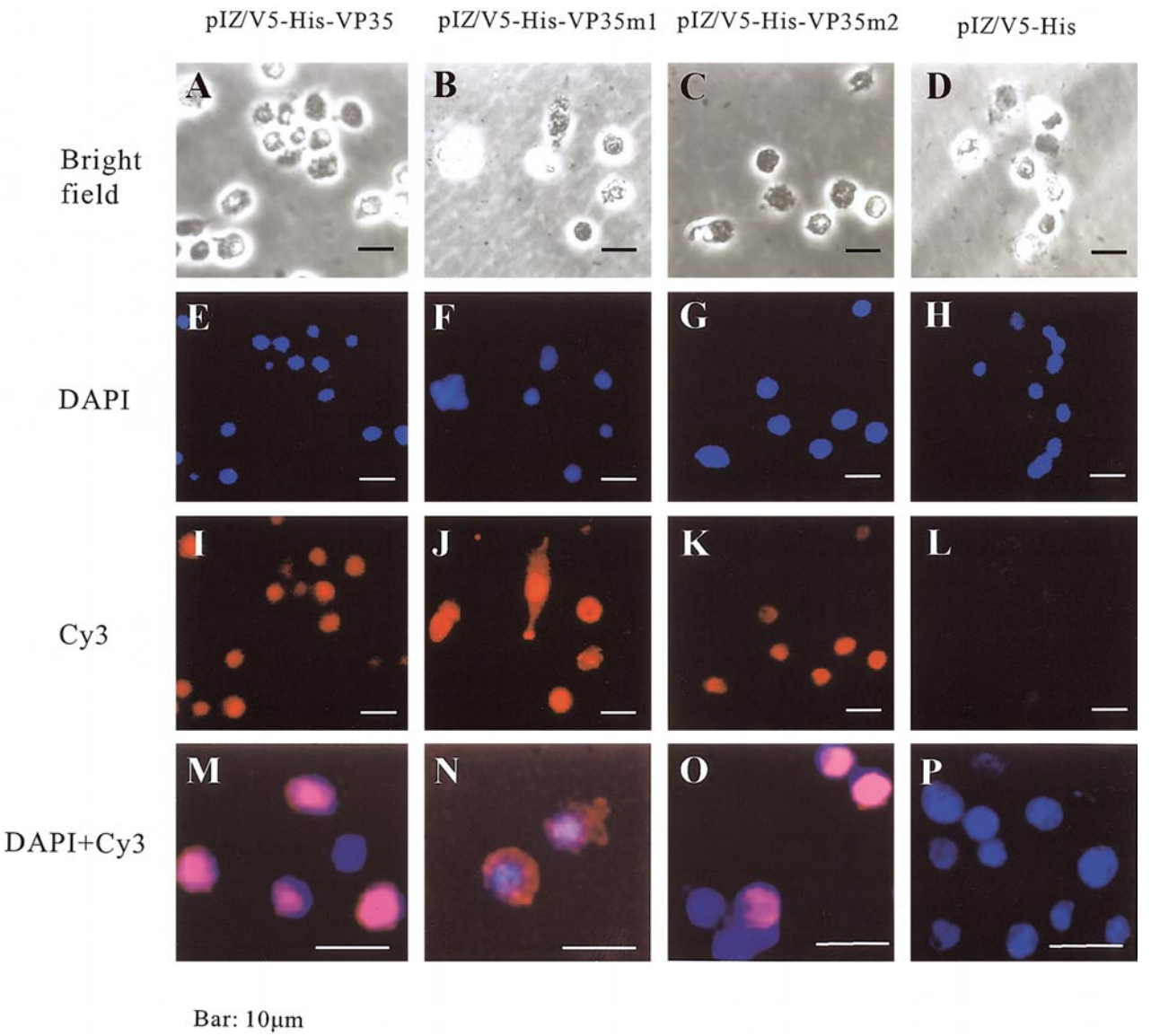

FIG. 9. Intracellular localization of normal VP35 and two NLS-mutant VP35 proteins in Sf9 cells. Sf9 cells were transfected with expression vectors pIZ/V5-His-VP35, pIZ/V5-His-VP35m1, pIZ/V5-His-VP35m2, and pIZ/V5-His, respectively, where the m1 mutant has AAAA instead of ^{24}KRKR^{27}, the m2 mutant has AAAA instead of ^{53}KRPR^{56}, and pIZ/V5-His is a negative control. Twenty-four hours after transfection, cells were stained with indirectly Cy3-conjugated antibody and counterstained with DAPI. The top row shows the transfected Sf9 cells under a bright field microscope, the second row shows the DAPI-counterstained nuclei of the same cells, the third row shows the Cy3 fluorescent signals from the transfected cells, and the last row shows the merged images of DAPI and Cy3 fluorescent signals from the transfected cells at higher magnification.

To engineer two mutant VP35s, in each of which one of the two potential NLS(s) (^{24}KRKR^{27} and ^{53}KRPR^{56}) was replaced with four alanines (AAAA), the genes of VP35 and the two mutant VP35 proteins were cloned into pIZ/V5-His vector to yield pIZ/V5-His-VP35, pIZ/V5-His-VP35m1, and pIZ/V5-His-VP35m2, respectively. The InsectSelect System was then used to transiently express VP35 and its mutants in Sf9 cells under the control of the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (*Op*MNPV) immediate-early gene promoter (OpIE2). The subcellular distribution of the resultant VP35 proteins was visualized 48 h after transfection by indirect immunofluorescence microscopy with a rabbit antibody against bacterially synthesized VP35 in combination with Cy3-conjugated goat anti-rabbit IgG antibody as a secondary antibody. Nuclei were stained with 4′-6′-diamidino-2 phenylindole dihydrochloride (DAPI) (Fig. 9). The transfection efficiency was about 80%. The Cy3 signals for the wild-type VP35 (Figs. 9I and 9M) and the m2 ^{53}AAAA^{56} mutant VP35 (Figs. 9K and 9O) were perfectly coincident with the nuclear DAPI signal, whereas expression of the m1 ^{24}AAAA^{27}-mutant VP35 (Figs. 9J and 9N) was spread throughout the cytoplasm of the transfected cells. This result implies that the sequence replaced in m1 mutant (i.e., ^{24}KRKR^{27}) is [a critical part of] the signal that causes nuclear accumulation of

VP35, that is, that the basic cluster formed by amino acids 24 to 27 is important for nuclear import of the protein.

VP35 is an acidic WSSV protein and it is also notable that an acidic amino acid-rich domain was observed near the NLS (^{24}KRKR^{27}) in VP35 (see Fig. 2). In several other proteins with an NLS, the acidic residues near the NLS have been shown to play an important role in the nuclear targeting of the protein (Makkerh *et al.,* 1996). Bearing this in mind, even though our present results (Fig. 9) show that mutation of the ^{24}KRKR^{27} sequence to alanines completely abolished the nuclear accumulation of the protein, we cannot be certain whether the ^{24}KRKR^{27} sequence constitutes the entire NLS signal itself or if it is a crucial part of a bigger signal.

In summary, Western analysis of the VP35 structural protein studied here revealed that it was associated with the nucleocapsid, and transient expression in insect cells (Sf9) showed that it targeted the nucleus. We defined and characterized a cluster of four positively charged residues at the N terminus of VP35 that directs the protein to the nucleus. When the four basic amino acids of this motif were replaced with AAAA, the mutant protein remained totally cytoplasmic, thus indicating its function as the nuclear localization signal of VP35. It still remains to be shown that VP35 actually serves to mediate the import of viral DNA into the nucleus, but meanwhile this report is the first description of an NLS found within a nucleocapsid protein of shrimp white spot syndrome virus.

MATERIALS AND METHODS

DNA sequencing and computer analysis

In 1994, WSSV was collected and purified from infected *Penaeus monodon* (Wang *et al.,* 1995) and then used to construct WSSV genomic libraries (Lo *et al.,* 1996). This virus source (known as the Taiwan isolate; Lo *et al.,* 1999) was used as the basis for all of the sequence work in the present study. Plasmid DNA for sequencing was purified using the QIAprep Miniprep System (Qiagen, Germany). The DNA fragments were sequenced by primer walking on both strands. The nucleotide and the predicted protein sequences were analyzed using GeneWorks 2.5.1 (Oxford Molecular Group, Inc., Campbell, CA). The DNA and the deduced amino acids sequences were compared with GenBank/EMBL, SWISSPORT, and PIR databases using the programs FASTA (Pearson and Lipman, 1988) and BLAST (Altschul *et al.,* 1997). The Prosite database was searched using Proscan (Bairoch *et al.,* 1997) and the hydrophobicity of VP35 was predicted according to the method published by Hopp and Woods (1981).

Virus inoculum preparation and experimental infection

WSSV inoculum was prepared from seriously infected (i.e., 1-step PCR positive; Lo *et al.,* 1996) *Penaeus monodon* shrimp. Carapace and integument tissue (0.5 *g*) was minced and then homogenized in 4.5 ml of sterile 0.9% NaCl solution. After centrifugation (1000 *g* for 10 min at 4°C), the supernatant was filtered through a 0.45-μm membrane and used immediately. Shrimps were infected with WSSV by intramuscular injection and tissues were collected at different times for analysis.

Virus purification

The virus used in this study was isolated from naturally infected *P. monodon* shrimp from Taiwan. It was first purified on a sucrose gradient as described previously (Wang *et al.,* 1995) and then further purified on a CsCl gradient (initially 40% in TE buffer, 10 mM Tris-HCl, 1 mM EDTA, pH 7.6) at 74,000 *g* at 10°C for 16 h. The visible viral band was then removed and pelleted by centrifugation at 74,000 *g* at 4°C for 30 min. The virus envelope was removed from the virus particles by adding Triton X-100 (1% in TE buffer) to the virus solution and incubating for 1 h at 37°C (Nadala *et al.,* 1998), and the nucleocapsids were then pelleted by centrifugation at 140,000 *g* at 4°C for 10 min. The pellet was dissolved in TE buffer for the SDS-PAGE and Western blot analysis.

Recombinant pms147 ORF5 protein (VP35) overexpression in *Esherichia coli,* antiserum production, and Western blot analysis

The DNA fragment encoding the full-length of WSSV VP35 (corresponding to ORF5 of pms147) was amplified from plasmid pms147 by PCR with the *vp35*FBamHI/R*Sal*1 primer set (GGATCCATGGTCTCTTCTAGAACA/GTCGACTTACCAACAAGGATCATC; the underlined bases indicate, respectively, the *Bam*H1 and *Sal*1 restriction sites that were used for subsequent cloning) and ligated to pGEM-T Easy (Promega). After confirming the sequence, the resultant plasmid, pGEM-T-VP35, was cleaved with *Bam*H1 and *Sal*1, and the amplified fragment was then cloned to pQE30 (Qiagen) at *Bam*H1 and *Sal*1 sites. The resultant recombinant plasmid, pQE30-VP35, was transformed into *Escherichia coli* JM109 strain. Liquid cultures were grown to an optical density of 0.6 at 600 nm and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C. The recombinant VP35 proteins tagged with six consecutive histidines (6× His-tagged VP35) were purified by using QIAexpressionist nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography (Qiagen) according to the manufacturer's recommendations. New Zealand white rabbits were hyperimmunized by injection with 100 μg proteins emulsified in complete Freund's adjuvant. Subsequent booster injections were carried out with 100 μg protein emulsified in

incomplete Freund's adjuvant. The antiserum was collected after the antibody titer had peaked.

For Western blotting analysis, proteins that had been separated in SDS-PAGE were transferred onto a polyvinylidene difluoride (PVDF) membrane (Micron Separations, Inc., Minnetonka, MN) by semidry blotting. Membranes were blocked in 5% skim milk (Difco Laboratories, Detroit, MI) in TBS (0.2 M NaCl and 50 mM Tris-HCl, pH 7.4). Immunodetection was performed by incubation of the blot in a polyclonal rabbit anti-VP35 serum diluted 1:2000 in TBS with 5% skim milk for 1 h at room temperature. Subsequently, anti-rabbit IgG antibody conjugated with horseradish peroxidase (Sigma, St. Louis, MO) was used at a concentration of 1:2000 and detection was performed with a Western Blot Chemiluminescence Reagent (NEN Life Sciences, Boston, MA).

Coupled *in vitro* transcription and translation

To facilitate detection of the HA-fusion protein in the *in vitro* transfection experiment (described below), an additional hemagglutinin (HA) tag was inserted into pcDNA3 (Invitrogen, The Netherlands). The DNA fragment encoding the full-length of WSSV VP35 (corresponding to ORF5 of pms147) was amplified from plasmid pms147 by PCR with the *vp35*FBamHI/R*Not*1 primer set (GGATCCATGGTCTCTTCTAGAACA/GCGGCCGCTTACCAACAAGGATCATC; the underlined bases indicate, respectively, the *Bam*H1 and *Not*1 restriction sites that were used for subsequent cloning) and ligated to pGEM-T Easy (Promega). After confirming the sequence, the resultant plasmid was cleaved with *Bam*H1 and *Not*I, and the amplified fragment was then cloned to pcDNA3-HA at *Bam*H1 and *Not*I sites to give pcDNA3-HA-VP35. Both autoradiography and Western blotting were used for VP35 analysis. For autoradiography, pcDNA3-HA-VP35 DNA (1 μg) was added to TNT Quick Master Mix (Promega) containing [^{35}S]methionine, and the mixture was incubated at 30°C for 60 to 90 min. For Western blotting, the same procedure was followed except that there was no [^{35}S]methionine labeling. After incubation, all samples were separated by 10% SDS-PAGE (Laemmli, 1970). In the autoradiographic procedure, the expressed proteins were visualized by exposure to X-ray film, while for Western blotting the proteins were detected by antibodies against bacterially synthesized pms147 ORF5 protein.

Recombinant pms147 ORF5 protein (rVP35) overexpression in insect cells

The BacVector Transfection system was used to construct the *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV)-based recombinant virus for the expression of VP35 in Sf9 cells. To this end, the full-length coding region of *vp35* was excised from the plasmid pQE30-VP35 with *Hin*dIII (filling-in with Klenow enzyme to generate a blunt end) and *Eco*RI and then cloned into the baculovirus transfer plasmid pVL1392 *Eco*RI/*Sma*I sites downstream of the polyhedrin promoter. The recombinant baculovirus was produced in Sf9 cells by cotransfection with the transfer plasmid (pVL1392-VP35) and linear viral DNA according to the BacVector Transfection Kit protocol (Novagen, Madison, WI). The purified recombinant baculovirus was then propagated in Sf9 cells at 27°C in Sf-900 II SFM cell culture medium (Gibco BRL, Frederick, MD). Forty-eight hours after infection, the infected cell proteins were separated by 10% SDS-PAGE and blotted onto PVDF membrane and then detected with antibodies against bacterially synthesized pms147 ORF5 protein.

Transcriptional analysis

Transcriptional analysis of *vp35* was performed by Northern blot analysis using total RNA isolated from artificially infected black tiger shrimp as described previously (Tsai *et al.,* 2000a). Briefly, for the isolation of total RNA, frozen tissues (500 mg) from WSSV-infected shrimps were homogenized in 5 ml TRIzol-LS reagent (Gibco BRL) and then subjected to 2-propanol extraction and ethanol precipitation according to the manufacturer's recommendations. The total RNA was stored in 75% ethanol at −70°C.

Total RNA in 75% ethanol was centrifuged at 14,000 *g* for 30 min at room temperature. The pellet was resuspended in DEPC-water and quantified by spectrophotometer. An aliquot of 10 μg RNA was treated with 200 U of RNase-free DNase I (Roche, Germany) at 37°C for 30 min to remove any viral genomic DNA contamination and then reextracted with phenol-chloroform. The DNase-treated total RNA was denatured by heating at 85°C for 10 min in 10 μl DEPC-water containing 100 pmol oligo-dT primer (Roche). The first strand cDNA was synthesized by the addition of 4 μl Superscript II 5× buffer, 1 μl 100 mM DTT, 1 μl 10 mM dNTPs, 10 U rRNasin (Promega), and 100 U Superscript II reverse transcriptase (Gibco BRL). The reaction proceeded at 37°C for 1 h. The cDNA reaction products were subjected to PCR with the primer set *vp35*-F/*vp35*-R (ATGGTCTCTTCTAGAACATC/TCACACTTGTGGAGCAACTGG) for the *vp35* gene. The β-actin gene served as an internal control for RNA quality and amplification efficiency as described previously (Tsai *et al.,* 2000a).

Detection of WSSV *vp35* transcripts in WSSV-infected shrimp by Northern blot hybridization analysis with a *vp35* gene-specific riboprobe

A WSSV *vp35*-specific [α-^{32}P]rCTP-labeled riboprobe was used for Northern blot analysis. To generate the riboprobe, the RNA polymerase promoter addition kit Lig'nScribe (Ambion, Austin, TX) was used in accordance with the manufacturer's instructions to produce templates from WSSV *vp35*-specific PCR products for the *in*

vitro transcription. Briefly, the WSSV *vp35*-specific fragment was amplified from WSSV genomic DNA by PCR with the primer set *vp35*-F/*vp35*-R. An aliquot (25 ng) of the WSSV *vp35*-specific PCR product was then ligated with T7 promoter adapter (supplied with the kit) using T4 DNA ligase. To generate WSSV *vp35*-specific fragments that contained the T7 RNA polymerase promoter, an aliquot (2 μl) of the reaction mixture (10 μl) was used as a template in PCR with a primer set consisting of the PCR adapter primer 1 (5′-GCTTCCGGCTCGTATGTTGTGTGG-3′, supplied with the kit) and *vp35*-F. An aliquot (3.6 μl) of PCR product (50 μl) was then used to generate the WSSV *vp35*-specific [α-^{32}P]rCTP-labeled riboprobe by *in vitro* transcription (Sambrook *et al.,* 1989) in a 20-μl reaction mixture containing 40 U T7 RNA polymerase (Roche) and 0.02 mCi [α-^{32}P]rCTP for 2 h at 37°C. The reaction mixture was then treated with 200 U RNase-free DNase I for 30 min at room temperature, terminated at 68°C for 15 min, and filtered through a Sephadex G50 column.

Total RNA (10 μg) was separated on 1% formaldehyde-agarose gel and transferred onto a Hybond-N+ membrane (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) (Sambrook *et al.,* 1989). The membrane was prehybridized for 1 h at 65°C in a prehybridization buffer (0.25 M phosphate buffer, 1 mM EDTA, 1% BSA, and 7% SDS) and then hybridized with a specific [α-^{32}P]rCTP-labeled riboprobe that was added to the buffer. After hybridization for 16 h at 65°C the membrane was washed for 5 min with wash buffer I (2× SSC, and 0.5% SDS) at room temperature, 30 min with wash buffer II (2× SSC and 0.1% SDS) at room temperature, and 30 min with wash buffer III (0.1× SSC and 0.1% SDS) at 65°C. The membrane was then exposed to Kodak BioMax MR film via an intensifying screen for several days at −70°C and the film was then developed.

Nuclear localization of WSSV VP35 in Sf9 cell culture, transfection, and immunofluorescent staining

The DNA fragment encoding the full-length of WSSV VP35 (corresponding to ORF5 of pms147) was amplified from plasmid pms147 by PCR with the vp35-pIZ-F1/vp35-pIZ-R1 primer set (AAGCTTATGGTCTCTTCTAGAACA/GGATCCCCAACAAGGATCATCAAT; the underlined bases indicate, respectively, the *Hin*dIII and *Bam*H1 restriction sites that were used for subsequent cloning) and ligated to pGEM-T Easy (Promega). After confirming the sequence, the resultant plasmid was cleaved with *Hin*dIII and *Bam*H1, and the amplified fragment was then cloned to pIZ/V5-His at *Hin*dIII and *Bam*H1 sites to give pIZ/V5-His-VP35. Four alanines (^{24}AAAA^{27}) were selected to replace the first NLS motif (^{24}KRKR^{27}) using overlap extension (Ho *et al.,* 1989). Two small fragments were amplified with the vp35-pIZ-F1/vp35-NLS1-R1 (TTCTGGCTCCGCCGCCGCCGCGGTGGGGAG) and vp35-pIZ-R1/vp35-NLS1-F1 (CTCCCCACCGCGGCGGCGGCGGAGCCAGAA) primer sets. One microliter of the two PCR products was supplied as templates for an additional PCR with the vp35-pIZ-F1/vp35-pIZ-R1 primer set. The final PCR product was then cloned into pIZ/V5-His to give pIZ/V5-His-VP35m1 as described above. The second NLS motif was mutated using the same method with primers vp35-NLS2-F1 and vp35-NLS2-R1 (TCAAGTAACGCGGCCGCCGCAATTAAGGAA and TTCCTTAATTGCGGCGGCCGCGTTACTTGA). The final PCR product was then cloned into pIZ/V5-His to give pIZ/V5-His-VP35m2. Cells were transfected with pIZ/V5-His-VP35, pIZ/V5-His-VP35m1, and pIZ/V5-His-VP35m2 at 70% confluency.

Sf9 cells were cultured at 27°C in Sf-900 II SFM (Gibco BRL). Transfection was performed in a 6-well format. About 10^6 cells were seeded in each well of the 6-well culture plate and cultured overnight. Plasmid pIZ/V5-His-VP35 and pIZ/V5-His-mVP35 (~2.5 μg) was mixed with Insectin-Plus liposomes (Invitrogen) in the medium and laid over the cultured cells according to the manufacturer's recommendations. Twenty-four hours after transfection, cells were fixed in 10% paraformaldehyde for 10 min at 4°C, treated with 0.1% Triton X-100 for 10 min at 4°C, washed extensively with PBS, and reacted with antiserum against VP35 at 4°C overnight. After washing, the cells were then reacted with carboxymethylindocyanine dye (Cy3) conjugated goat anti-rabbit IgG antibody (Sigma). Counterstaining of the nucleus was performed with 4′-6′-diamidino-2 phenylindole dihydrochloride (DAPI, Vector Laboratories, Burlingame, CA). Cy3 and DAPI were excited by light at 552 and 372 nm, respectively, and fluorescence was observed (at 565 nm for Cy3 and 456 nm for DAPI) with an Olympus IX70 microscopy system with a fluorescent microscopy apparatus.

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